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Guidance on the assessment of the efficacy of feed additives

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Abstract

This guidance document is intended to assist the applicant in preparing and presenting an application, as foreseen in Article 7.6 of Regulation (EC) No 1831/2003, for the authorisation of additives for use in animal nutrition. It specifically covers the assessment of the efficacy of feed additives.

KEYWORDS

efficacy, feed additives, guidance

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1 | INTRODUCTION

1.1 | Background and Terms of Reference

Regulation (EC) No 1831/2003 establishes the rules governing the Community authorisation of additives for use in animal nutrition. Moreover, Regulation (EC) No 429/2008 provides detailed rules for the implementation of Regulation (EC) No 1831/2003 as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives. The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP Panel) has adopted a series of Guidance documents which aim at complementing Regulation (EC) No 429/2008 to the applicants in the preparation and submission of technical dossiers for the authorisation of additives for use in animal nutrition according to Regulation (EC) No 1831/2003. At the plenary meeting in September 2021, the FEEDAP Panel identified the following Guidance documents and statement for revision:

- the Guidance on user safety, considering recent scientific developments and the Panel's experience gained during the last years while working under the provisions of Regulation (EC) No 429/2008,
- the Guidance on the assessment of the efficacy of feed additives, making it complementary to the revised Regulation (EC) No 1831/2003 by stimulating innovation and sustainability, in particular for additives that are beneficial for the environment and animal welfare, as outlined in the Green Deal,
- the Guidance on the characterisation of microorganisms used as feed additives or as production organisms, harmonising it with related EFSA Guidance documents, and
- the EFSA Statement on the requirements for whole genome sequence analysis of microorganisms intentionally used in the food chain, keeping track of the fast development in this field.

In view of the above, the European Food Safety Authority (EFSA) asks its FEEDAP Panel to:

1. Analyse for the identified Guidance documents which aspects are most relevant to be updated based on the scientific developments and stakeholder perspective.
2. Update the identified Guidance documents, focussing on the most relevant aspects and taking into account the comments received during public and/or targeted consultations.

This document addresses the terms of reference regarding the Guidance on the assessment of the efficacy of feed additives.

In line with EFSA's policy on openness and transparency, and for EFSA to receive comments from the scientific community and stakeholders, a draft of the Guidance was released for public consultation.[1] The outcome of the public consultation is described in a technical report published as Annex A[2] to this Guidance.

1.2 | Scope of the guidance

This guidance document is an update of the EFSA FEEDAP Panel Guidance on the assessment of the efficacy of feed additives published in 2018 (EFSA FEEDAP Panel, 2018a), which it replaces. Like the previous version, it is intended to assist the applicant in preparing and presenting its application for authorisation of a feed additive, as foreseen in Article 7.6 of Regulation (EC) No 1831/2003. This document does not substitute for the obligation of an applicant to comply with the requirements of Regulation (EC) No 1831/2003 and its implementing rules (Commission Regulation No 429/2008). This document is intended to guide applicants in assessing the efficacy of additives intended to be used in animal feed to demonstrate compliance with the requirements of Article 5.3 of Regulation (EC) No 1831/2003. This Guidance is divided into seven sections. Section 2 provides the principles of the assessment of efficacy. The requirements for efficacy demonstration for the different categories of additives are listed in Section 3. Section 4 provides information on the number of efficacy studies required for those additives for which in vivo studies are needed. Sections 5 and 6 describe the principles for in vivo and in vitro studies, while Sections 7 and 8 provide information on how to report the studies performed by the applicant or those retrieved from the literature.

Applicants should justify the omission from the dossier of any data or any deviations from the requirements detailed in this Guidance.

2 | GENERAL PRINCIPLES OF EFFICACY ASSESSMENT

Regulation (EC) No 429/2008 requires that studies should demonstrate the efficacy for each proposed use and satisfy at least one of the characteristics set out in Article 5(3) of Regulation (EC) No 1831/2003, according to the categories and functional groups of feed additives as provided by Article 6 and Annex I of the said Regulation. Moreover, such studies must permit the evaluation of the efficacy of the additive according to common feed manufacturing, animal husbandry, farming practices and animal welfare rules in the European Union (EU). Studies performed outside the EU must permit conclusions

[1] https://connect.efsa.europa.eu/RM/s/consultations/publicconsultation2/a0lTk0000001MZZ/pc0735

[2] Annex A can be found in the online version of this output (in the 'Supporting information' section).

to be drawn on the efficacy of the additive when used in the EU. Any potential impact on the distinctive features of animal products should also be investigated during animal efficacy trials (e.g. off-flavour, colour changes).

All efficacy studies submitted should be properly reported and documented to allow an adequate assessment. The applicant is encouraged to standardise study protocols and reports as far as possible to facilitate the comparison of results. The studies should be based on the additive(s) for which authorisation is sought or, if not, should still allow conclusions on the additive under application to be drawn. To avoid confusion, in-house identifiers should be avoided unless embedded in third-party documents. In this case, a statement is required to confirm that the identifier(s) refers to the additive(s) concerned.

However, the Panel considers that there are some additives for which efficacy is recognised (e.g. many nutritional additives and flavouring compounds). These additives do not require further demonstration of efficacy. For others, it is not practical to assess the efficacy of the additive under all possible conditions of use. Many factors may affect the efficacy of an additive, e.g. nutrition, animal breeds, composition of feed, management, environment and husbandry. For such additives, the Panel can conclude on the efficacy under the conditions of the studies submitted. From these data, the Panel may be able to conclude on the potential efficacy of the additive under EU farming conditions.

As a general principle, efficacy can be assessed using in vitro studies for additives intended only to affect the characteristics of feed (i.e. some technological and sensory additives). In contrast, for those intended to have an effect in the animal, efficacy should be assessed via in vivo studies or, in specific circumstances, by a combination of in vitro and in vivo studies. The number of studies required to support the efficacy of an additive will depend on the nature of the intended effect(s) and the conditions of use of the additive (e.g. target species/categories). Efficacy should be investigated by comparison of the lowest recommended use level (in feed or water) with an appropriate control group and designed to allow statistical evaluation.

Efficacy should be supported by independent studies showing statistically significant effects on key endpoints related to the expected effect of the additive.

Reference can be made to published studies to fulfil the requirements listed in the Guidance provided that the active substance/agent in literature studies is identical to that under application or, if not, would still allow conclusions on the additive under application to be drawn.

Attention should also be paid to known potential biological or physicochemical interactions between the additive, other additives, veterinary medicines and/or components of the diet, where this is relevant to the efficacy of the additive concerned, e.g. compatibility of a microbial additive with coccidiostats and histomonostats, or organic acids. For details on how to perform compatibility studies between microbial additives and other additives showing antimicrobial activity, see the Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018b).

Studies involving animals should respect the rules on animal welfare laid down by the EU legislation, particularly those listed in Directive 63/2010/EU.

3 | REQUIREMENTS FOR THE DIFFERENT CATEGORIES OF ADDITIVES

3.1 | Technological additives

When the additive is already authorised for use in food and the intended use of the additive in feed is the same, no further demonstration of efficacy is generally necessary, provided that the effect seen when the additive is used in food could reasonably be expected to be seen when it is used in feed at the recommended concentration, and that food and feed matrices are comparable. Evidence that food and feed matrices are comparable should be provided.

3.1.1 | Technological additives which exert their function in feed

For technological additives intended to affect the characteristics of feed, efficacy should be demonstrated using laboratory-based studies by means of appropriate criteria as reflected in recognised acceptable methods under the intended practical conditions of use in comparison with an appropriate control feed.

The studies (at least three) should be designed to cover a representative range of feeds to which the additive will be applied.

The appropriate endpoints for the demonstration of the efficacy of the various functional groups are indicated in Table 1.

TABLE 1 Endpoints for the demonstration of the efficacy of technological additives exerting their effect in feed.

Functional group	Endpoints for the demonstration of efficacy
Preservatives	Inhibition of the growth of spoilage microorganisms. The duration of the study should cover the period for which an effect is claimed. Test materials could be naturally or artificially contaminated.
Antioxidants	Protection against oxidative damage of key nutrients/components during feed processing and/or storage. The period for which a protective effect is claimed should be demonstrated.
Emulsifiers	Formation/maintenance of stable emulsions of otherwise immiscible or poorly miscible feeds.
Stabilisers	Maintenance of the physico-chemical state of feeds, including the use of coating agents.
Thickeners	Viscosity of the feeds.
Gelling agents	Formation of a gel resulting in a change in the texture of the feeds.

(Continues)

TABLE 1 (Continued)

Functional group	Endpoints for the demonstration of efficacy
Binders	Pellet durability (hardness, abrasion) or energy consumed during pellet formation.
Anti-caking agents	Flowability (angle of repose, frictional forces, compressibility).
Acidity regulators	pH and/or buffering capacity in feeds.
Silage additives	Improved production/quality of silage (better preservation of nutrients). Inhibition of undesirable microorganisms. Reduction of effluents. Improved aerobic stability.
Denaturants	Indelible identification of feeds.
Hygiene condition enhancers	Reduction of contamination with specific microorganism(s) relevant to feed safety (e.g. potential human or animal enteropathogens or undesirable bacteria).
Other technological additives	The endpoints used to assess the function/effect of the additive should be defined and justified.

3.1.1.1 | *Silage additives*

The applicant should clearly indicate the substrate to which the additive is intended to be used (e.g. dry matter range and the water-soluble carbohydrate content, botanical origin).

For additives intended for the preparation of silage from all fresh plant feed materials, a minimum of three separate tests should be made, including one example of each of the following categories, where possible, using material of different botanical origins:

- Easy to ensile: > 3% soluble carbohydrates in the fresh material.
- Moderately difficult to ensile: 1.5%–3.0% soluble carbohydrates in the fresh material.
- Difficult to ensile: < 1.5% soluble carbohydrates in the fresh material.

For additives intended for the preparation of silage from specific sub-categories of fresh material, three tests should be made with materials representative of the claimed range, where possible, using material of different botanical origins.

Claims restricted to or including feeds other than plant material require tests specific to those feeds (e.g. fish silage).

All studies should demonstrate efficacy in comparison to a negative control made with the same material for ensiling but without the additive.

As a general guide, all replicate tests should be made with at least 1 kg of homogeneous fresh material in a closed laboratory silo with the potential to vent gas and, when a claim for effluent reduction is made, to drain effluent. Other test systems (e.g. wrapped bales, vacuum-packed bags) may be used, provided they are consistent with the claims made and meet the general requirements above (including negative controls). The harvesting and preparation of the test material must be similar to standard practice. Methods used in the preparation of the silos, including compaction, should be the same across replicates. The duration of each study should be at least 90 days at a constant temperature (recommended range 15–25°C).

Claims made for silage additives differ and may relate to the preservation process in general, to specific aspects of the preservation process, or to the aerobic stability of silage once the clamp/silo has been opened. The observations needed to demonstrate a significant benefit for the proposed lowest additive concentration in the material to be ensiled will differ in nature, sampling time and frequency. As a rule, measurements of the following parameters should be provided in comparison to the negative control:

- total dry matter content (corrected for the loss of volatile compounds[3])
- calculated dry matter losses during ensiling
- pH
- volatile fatty acids and lactic acid (including the calculated lactic acid to acetic acid ratio)
- alcohols
- ammonia nitrogen

In addition, other microbiological and chemical parameters should be included as appropriate to substantiate the specific claim (e.g. clostridia, *Listeria* in silage for sheep).

The improvement of the silage production/quality or the preservation of nutrients should be demonstrated by a positive/favourable effect on at least two of these endpoints in each study, one of them being either reduced dry matter loss or lower ammonia nitrogen content in silage.

[3]Depending on the drying method applied, some volatile compounds may be lost, affecting the accuracy of the DM content determined in the silage. The procedure used to estimate the silage DM content corrected for the loss of volatiles should be duly referenced and justified considering the method used for DM determination (e.g. drying temperature) and the type of silage. The calculations performed should be made available.

A claim for effluent reduction will be judged against the total volume of effluent produced over the entire experimental period, considering the likely effect on the environment (e.g. ecotoxicity of the effluent, biological oxygen demand). Reduction of effluent production should be demonstrated directly. The duration of the study should be at least 50 days.

For claims related to the improvement of the aerobic stability of silage after opening the silo, the duration of the test should be at least 7 days after exposure to air, and the additive should provide evidence of stability for at least 2 days longer than that shown by the untreated control. It is recommended that the experiment is conducted at an ambient temperature of 20°C, and a rise in temperature of 3°C or more above the background is taken as indicative of instability.

3.1.1.2 | *Hygiene condition enhancers and/or preservatives*

The following principles would apply to hygiene condition enhancers and preservatives. The choice of the target microorganism(s) would depend on the functional group for which the application is made (spoilage microorganisms for preservatives and microorganisms relevant to feed safety for hygiene condition enhancers).

Evidence of efficacy should be demonstrated using a minimum of three independent in vitro studies. The choice of the feeds and the target microorganism(s)/contaminant(s) against which the additive will exert its function should be justified and reflect the proposed conditions of use. Proper endpoints should be measured in the feeds containing the additive in comparison with an appropriate control.

For additives intended to be used in all feeds, efficacy should be demonstrated in a representative range of feeds and dry matter content according to the intended use (i.e. covering a range of approximately 10%–80% DM). The pH, dry matter content and water activity of the matrix should be provided for each study.

For hygiene condition enhancers, the choice of the target microorganism(s) should cover several unrelated reference/well-known strains and field strains (e.g. environmental/feed isolated strains). Data should be provided to confirm taxonomic/serotype identification and to exclude clonality. At least four replicates for each strain of the target microorganism(s) tested should be included in each experiment. This would also apply to preservatives when the feed is inoculated with spoilage microorganisms.

For hygiene condition enhancers, different molecular (sero)types relevant for humans and the target animals should be tested (e.g. for *Salmonella* spp. at least four serovars to reach a minimum of five strains, one of which should be a reference or well-known strain; for a specific serovar, e.g. *S*. Typhimurium, at least three strains should be tested, one of which should be a reference strain). In all cases, a clear description of the rationale for selecting the type and number of strains/serovars should be provided based on the claimed effect of the additive, the mode of action of the active substance/agent and the conditions of use.

The experimental design should include at least two groups: one with the feed contaminated with the target microorganism(s) (control) and another with the same contaminated feed supplemented with the additive at the minimum use level. If appropriate, other groups with different levels of the additive may be included in the design.

Studies can be done with naturally or artificially contaminated feeds. A range of the target microorganism(s) of 10^2–10^4 colony forming units (CFU)/g is needed. In the case of artificially contaminated feed, the additive and target microorganism(s) can be added simultaneously for both hygiene condition enhancers and preservatives; in those cases, the selection of the inocula and the experimental conditions (e.g. inoculum preparation, inoculation technique) should be justified.

Feed(s) samples should be incubated reflecting practical farming conditions (e.g. in the presence of atmospheric oxygen, ambient temperature and humidity). Other practical use conditions should be detailed and reflected in the study design (e.g. constant mixing). Pre-treatments not routinely used in feed production/preparation and intended to reduce the background microbial contamination of the experimental feed (e.g. sterilisation of the feeds, use of antimicrobial substances) should be avoided.

The duration of the study should reflect actual use conditions and cover the period for which an effect is claimed according to the proposed conditions of use. Sampling should include measurements at the start and end of the study and at least one intermediate time point to establish/define the beginning of the effect (i.e. reduction of the contamination).

In the case of hygiene condition enhancers, feed samples should be monitored for the viable counts of the specific target microorganism(s). In the case of preservatives, microbial groups (e.g. total numbers of yeasts, fungi and aerobic bacteria) should be analysed using cultivation-based methods. Evidence of adequate sampling should be provided. Efficacy is demonstrated if the counts of the target microorganism(s) at the end of the experiment are statistically lower in the supplemented feed compared with the control, and this difference is biologically relevant. Changes in microbial counts below 0.5 log are considered within the normal variation of the methods and will not be taken as proof of an effect.

The microbial quality of the feed should be evaluated at least at the start and the end of the study (e.g. pH, temperature, counts of total aerobic bacteria, Enterobacteriaceae, total yeasts and filamentous fungi). For target microorganisms producing toxic compounds, the presence of these compounds should be analysed in the feed samples at the end of the study.

In the case of additives based on microorganisms, any pre-treatments not routinely used in the feed production/preparation and intended to precondition or stimulate the growth of the active agent(s) present in the additive (e.g. use of buffers and/or nutrient broths and incubation conditions optimal for the strain(s) used as an additive) but not relevant for the feed production, should be avoided.

3.1.2 | Technological additives which exert their function in the animal

The intended effects of 'Substances for the reduction of contamination of feed by mycotoxins' and 'Substances for control of radionuclide contamination' are not expected to be observed until feed containing the additive is fed to the animal. Therefore, the demonstration of efficacy should be based on in vivo studies.[4]

The appropriate endpoints for the two functional groups are indicated in Table 2.

TABLE 2 Demonstration of efficacy for technological additives exerting their effect in the animal.

Functional group	Demonstration of efficacy
Substances for the reduction of contamination of feed by mycotoxins	Reduction of the absorption of mycotoxins. Increased excretion of mycotoxins. Degradation/transformation of mycotoxins. Reduced concentration of mycotoxins in food of animal origin.
Substances for control of radionuclides	Evidence of reduced contamination of food of animal origin.
Other technological additives	The endpoints used for assessing the functionality of the additive should be defined and justified.

3.1.2.1 | *Substances for reduction of the contamination of feed by mycotoxins*

The mycotoxin(s) against which the additive will exert its function and the target species should be specified.

In vitro studies can provide evidence of the intended effect of the additive reducing the contamination by mycotoxins. However, in vitro studies do not sufficiently mimic the conditions in the digestive tract and the differences between target animals and their metabolism to fully demonstrate efficacy under practical conditions. Therefore, in vitro studies showing the effects of the additive should be supported by in vivo studies.

A minimum of three independent in vivo studies (generally short-term) showing relevant and significant effects should be provided to demonstrate efficacy at the lowest recommended dose (see Section 5 for recommendations for the design of in vivo studies).

For additives intended to be used in two target species, at least three studies covering the different target species and categories covering both growing and reproductive animals, as relevant, should be submitted. For example, for an application covering all poultry and all porcine species, one study in chickens for fattening, one in weaned piglets and one in sows or laying hens would suffice. For additives intended to be used in all terrestrial species, efficacy should be demonstrated in vivo in three major species (at least one study in each) representing different digestive systems (a poultry species, a pig and a ruminant). For additives intended to be also used in fish, at least one additional study in salmonids is required.

In each case, the studies should include the animal category for which the lowest maximum content of the respective mycotoxin in feed is set in Directive 2002/32/EC[5] or recommended in Commission Recommendation 2006/576/EC.[6]

The target mycotoxin content in feed used in studies should not exceed the values given in Directive 2002/32/EC for aflatoxin B_1 and in Commission Recommendation 2006/576/EC for deoxynivalenol, zearalenone, ochratoxin A and fumonisins B1 + B2 for complete feeds for the respective animal species/category and Commission recommendation 2013/165/EU[7] for T-2 and HT-2. For mycotoxins without a maximum content established at the EU level, the dietary levels chosen should not exert adverse effects on the target animals.

Naturally contaminated feeds are preferred as the source of mycotoxins. Alternatively, feed spiked with mycotoxins could be used if properly justified. For each trial, a quantitative analysis of mycotoxins[8] present in the feed should be provided. Evidence of adequate sampling should we provided.

The experimental design of the studies should include at least two groups: one fed the basal contaminated diet as such (control) and the other fed the same basal contaminated diet supplemented with the additive for which authorisation is sought. For mycotoxins without a maximum content set/recommended, an additional control group should be included to ensure no adverse effects at the concentrations used. In this group, the feed should be free of these mycotoxins[9] and have, in general, the same composition as the feed given to the other two groups.

In general, mycotoxin/metabolites excretion in faeces/urine, concentration in blood/plasma/serum, tissues or products (milk or eggs) or other relevant biomarkers should be taken as endpoints for demonstration of efficacy. The endpoints should be selected according to the mycotoxin and target species/categories, taking into account the availability of sensitive analytical methods validated for the specific matrices. Recommendations on the endpoints for each specific mycotoxin are given in Table 3.

[4]In case in which the effect is expected before the ingestion by the animal (e.g. in silage), in vitro studies may be sufficient to support the efficacy of the additive.

[5]Directive 2002/32/EC of the European Parliament and of the council of 7 May 2002 on undesirable substances in animal feed.

[6]Commission Recommendation of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding.

[7]Commission Recommendation of 27 March 2013 on the presence of T-2 and HT-2 toxin in cereals and cereal products.

[8]Including at least aflatoxin B_1 and B_2, deoxynivalenol, nivalenol, zearalenone, ochratoxin A, fumonisins B1 + B2, T-2 and HT-2, and any other for which a claim is made should be determined.

[9]Below or at least close to the limit of detection.

Zootechnical parameters should be reported but cannot be used to demonstrate efficacy.

TABLE 3 Most relevant endpoints/biomarkers for substances reducing the contamination of feed by mycotoxins.

Target mycotoxin(s)	Most relevant endpoints
Aflatoxin B_1	Aflatoxin M_1 in milk/egg yolk
Deoxynivalenol	DON/metabolites in blood serum
Zearalenone	Zearalenone + α- and β-zearalenol in plasma Excretion of zearalenone/metabolites via faeces/urine
Ochratoxin A	Ochratoxin in kidney (or blood serum)
Fumonisins B1 + B2	Sphinganine/sphingosine ratio in blood, plasma or tissues

3.1.2.2 | *Substances for control of radionuclide contamination*

For substances for control of radionuclide contamination, a similar approach to the one for substances for reduction of the contamination of feed with mycotoxins should be followed. However, a single study demonstrating positive effects would generally suffice to support the efficacy.

3.2 | Sensory additives

When the additive is already authorised for use in food and the intended use of the additive in feed is the same, no further demonstration of efficacy is generally necessary, provided that the effect seen when used in food could reasonably be expected to be seen when used in feed at the recommended concentration, and that food and feed matrices are comparable.

3.2.1 | Substances that add or restore colour in feeds

Evidence of the efficacy of the additive should be demonstrated using laboratory-based studies by means of appropriate criteria as reflected in recognised acceptable methods under the intended practical conditions of use in comparison with an appropriate control feed. The change in the colour of feeds should be measured using appropriate methodologies (e.g. reflectance spectroscopy, image analysis). The studies (at least three) should be designed to cover a representative range of feeds to which the additive will be applied. The additive should not adversely affect feed quality.

3.2.2 | Substances which, when fed to animals, add colour to food of animal origin

A minimum of three independent in vivo studies showing significant effects should be provided to demonstrate efficacy for the relevant target species/categories.

Reference to published studies can be provided if the relationship between a particular substance and the colour of animal tissues/products is well documented.

Evidence should generally be provided for each target species/category for which the application is made. The change in colour of tissues/products obtained from animals receiving the additive should be measured using appropriate methodologies (e.g. colour fan, reflectance spectroscopy, image analysis).

3.2.3 | Substances which favourably affect the colour of ornamental fish and birds

Evidence of efficacy can be provided by:

(i) reference to published studies where the relationship between a particular substance and the colour of the animals has been established,
(ii) extrapolation of the colouring effect established in poultry or salmonids, as appropriate,
(iii) in vivo studies in the target species.

For (i) or (iii), a minimum of three independent long-term in vivo studies showing significant effects should be provided. The change in colour of animals receiving the additive should be demonstrated.

3.2.4 | Flavouring compounds

Evidence of efficacy can be provided by:

(i) reference to literature,
(ii) laboratory-based studies (e.g. sensory panel, electronic nose)
(iii) short-term in vivo studies if the application includes an effect on palatability.

For (iii), for applications for one single animal species/category, a minimum of three independent studies showing relevant and significant effects should be provided. For applications for multiple animal species/categories, the minimum number of studies should follow the requirements defined in Section 4.

3.3 | Nutritional additives

No evidence of efficacy is necessary for amino acids naturally occurring in proteins of plants and animals and their salts, urea and vitamins, pro-vitamins and compounds of trace elements already assessed and authorised under Regulation (EC) No 1831/2003.[10]

Evidence of efficacy should be provided for amino acid analogues, new forms of compounds of trace elements, chemically well-defined substances having similar effects to vitamins and urea derivatives. Evidence can be provided by reference to literature or by in vivo studies. Where evidence from the literature is insufficient to conclude:

- For amino acid analogues, urea derivatives and new forms of compounds of trace elements, one bioavailability or one bioequivalence study is considered adequate to demonstrate efficacy.[11]
- For chemically well-defined substances having similar effects to vitamins, the choice of short-/long-term studies and the endpoints to be measured will depend on the nature of the substance and the intended effect.
- For other nutritional additives, at least one long-term efficacy study should be provided.

Generally, one study in a single animal species or category, including laboratory animals, will be sufficient to demonstrate efficacy. For additives specifically designed to be efficacious in a particular animal species/category (e.g. protected amino acids for ruminants), the same target species should be selected.

3.4 | Zootechnical additives

Generally, a minimum of three independent in vivo studies showing relevant and significant effects should be provided to demonstrate efficacy for the relevant target species/categories. Efficacy studies should always include the lowest incorporation level (e.g. mg/kg complete feed or water)/lowest daily level (e.g. mg/head per day) of the additive proposed by the applicant. Depending on the nature and the expected effect of the additive, short-term trials, long-term trials, or a combination of short- and long-term trials would be needed to demonstrate the efficacy.

In contrast with other categories of additives in which there is a link between the functional group(s) and the mode(s) of action, for zootechnical additives, the assessment will be performed exclusively based on the effect(s) expected of the additive in line with Article 5.3 (b), (e) and (f) of Regulation (EC) No 1831/2003.

3.4.1 | Additives affecting animal production or performance

For additives affecting animal production or the performance of animals, long-term efficacy studies should be provided unless the use of the additive/active substance is restricted to specific short-term periods for which particular provisions apply (see Section 5.2). Depending on the properties of the additive, outcome measures may be based on performance or reproduction parameters.

Only in the case of enzymes affecting the utilisation of phytate phosphorus or the digestibility of polysaccharides or proteins, short-term studies can substitute long-term studies, provided that adequately defined and specific methods are applied:

- For phytases: improved utilisation of dietary phosphorus by total P retention in balance trials or P digestibility plus partial P (bone) retention.
- For polysaccharidases: increased metabolisable energy in balance trials.
- For proteases: improved utilisation of dietary protein by nitrogen retention in balance trials or by ileal digestibility of amino acids.

Apparent faecal digestibility studies alone are not considered sufficient to demonstrate the efficacy of these enzymes. The only exception is cases where balance trials are not recommended for technical reasons, such as studies in cows or sows.

3.4.2 | Additives favourably affecting the environmental consequences of animal production

For additives which favourably affect the environment by direct or indirect means, in vivo studies showing significant effects should be provided to demonstrate efficacy in the relevant target species/categories.

[10]Applications for vitamins, pro-vitamins and compounds of trace elements should consider the classification of these substances by the scientific community.

[11]See Section 5.2.1.1 Bioavailability/bioequivalence studies, Section 5.1.3 Experimental groups and Section 5.1.6 Statistical considerations for more details on the experimental design of this type of trials.

- For additives aimed at reducing the output of nitrogen, phosphorus or trace elements to the environment (e.g. by allowing the use of diets with lower nitrogen or phosphorus content), short-term (balance) studies can substitute for long-term studies, provided that adequately defined and specific methods are applied.
- For additives aimed at reducing the production of methane and other greenhouse gases, the reduction in gas production should be measured by internationally recognised methods in long-term studies showing the persistency of the effects. The studies should assess the potential effects of the additive on zootechnical parameters and consider the possibility of an adaptive response to the additive (different timepoints should be considered).
- For additives intended to reduce odour, efficacy demonstration should be based on objective methods to monitor odour emission (e.g. sensory panel, electronic nose, analysis of marker odour compounds). Evidence can be provided by short-term studies, but at least one long-term study should be performed to consider the possibility of an adaptive response to the additive.
- For other effects, the intended effect of the additive should be clearly specified, and the selection of endpoints adequately justified.

3.4.3 | Additives affecting the characteristics of food of animal origin

For additives affecting the characteristics of food (other than sensory additives which affect the colour of food), the choice of long-term or short-term studies to demonstrate the efficacy of these additives will depend on the nature of the substance and intended purpose. The selection of the endpoints should be adequately justified.

3.4.4 | Additives affecting animal welfare

For additives favourably affecting welfare, the choice of long-term or short-term studies will depend on the nature of the substance and the intended purpose. The selection of the endpoints should be adequately justified.

For additives affecting stress resilience, it might be possible to apply stressing factors representing realistic situations of the animals' life/productive cycle (e.g. heat stress, transport, husbandry systems), which may challenge their optimum physiological status and/or welfare (e.g. physiology, behaviour, affective state). In those cases, a clear description of the rationale for selecting the stressor(s) and the endpoints monitored should be provided a priori based on the stressor applied, the claimed effect of the additive and the conditions of use. The choice of long-term or short-term studies to demonstrate the efficacy will depend on the nature of the substance and intended purpose. The experimental design should include at least two groups: one unsupplemented control group and one group supplemented with the additive, both exposed to the same challenge/stressor. The efficacy of the additive will be assessed by comparison of these two groups.

The link between the stressor and the welfare implication (i.e. welfare consequence[12]) for the animal should be demonstrated by in vivo studies using physiological, behavioural and/or immunological endpoints. Justification for using a particular endpoint to assess the welfare implication, including, when possible, information on the sensitivity, specificity and feasibility of the endpoint should be provided.

3.4.5 | Other effects

The intended effect of the additive should be clearly specified. The choice of long-term or short-term studies to demonstrate the efficacy of other additives under the category of zootechnical additives will depend on the nature of the substance and its intended purpose. The selection of the endpoints should be adequately justified.

3.5 | Coccidiostats and histomonostats

These additives are intended to protect animals from the consequences of an invasion of *Eimeria* spp. or *Histomonas meleagridis* by killing or inhibiting these protozoa. The text below provides Guidance for assessing the efficacy of coccidiostats in poultry and rabbits. The requirements below should be adapted and justified for applications covering other animal species or histomonostats.

The capacity of anticoccidial substances to control coccidiosis should be demonstrated by targeting specific endpoints (e.g. lesion/faecal score, oocyst excretion, morbidity, coccidiosis-related mortality). Data on zootechnical performance should be provided as supporting information.

Efficacy data, testing the minimum inclusion levels, should derive from two types of target animal experiments using artificial infection:

- Floor pen studies with poultry or battery cage studies with rabbits
- Anticoccidial sensitivity tests (AST)

[12]Welfare consequence: the change in welfare that results from the effect of a factor or factors (EFSA AHAW, 2012). List of negative welfare consequences and overarching affective states can be found in EFSA AHAW (2022) as example.

3.5.1 | Floor pen studies with poultry/battery cage studies with rabbits

For floor pen studies with poultry and battery cage studies with rabbits, three studies are required with inocula from different regions within the EU that are sufficiently distant to guarantee that the *Eimeria* strains are not related. Evidence should be provided that the inocula used in the studies are different. The studies should be conducted not more than 5 years before the date of submission of the application. A negative control (without a coccidiostat) is essential. The design of such a study usually consists of three groups:

- uninfected untreated control (UUC)
- infected untreated control (IUC)
- infected treated (IT)

A fourth optional group may be included:

- uninfected treated (UT)

The study duration should be that required for long-term efficacy studies (see Section 5.2.2). The different endpoints should be measured at least at 5–7 days (poultry) or 4–7 days (rabbits) after inoculation (depending on the prepatent period of *Eimeria* species present in the inoculum), while the intestinal lesions should also be measured at day 14 after inoculation and at the end of the study. It is recommended to expose all animals in the IUC and IT groups to the inoculum and not rely on seeder animals.

3.5.2 | Anticoccidial sensitivity tests

Three anticoccidial sensitivity studies are required, with inocula from different regions within the EU that are sufficiently distant to guarantee that the *Eimeria* strains are not related and show significant and positive results. The studies should be conducted within 2 years before the submission of the application. If one study is performed in the same location and uses a mutual UUC group but tests inocula from multiple sources, separate statistical analyses should be performed for each inoculum.

Sensitivity tests should be performed according to the principles established by Chapman (1998) and following the guidelines published by Holdsworth et al. (2004) and Cervantes and McDougald (2022).

For poultry, birds should be fed the same basal diet until grouping, when the experimental diets should be introduced. The allocation of replicates to treatment groups should be done 1 or 2 days before inoculation at days 13–16 of age. Examination of endpoints should generally be done 5–7 days after inoculation. Zootechnical parameters should be reported for this experimental period (from grouping until completion).

In the case of rabbits, a protocol similar to the one described above for poultry should be applied.

3.5.3 | Inocula

The inoculum is the critical factor in the models used in studies with artificial infection. The inoculum (sporulated oocysts) should represent EU field coccidia strains exposed to currently approved coccidiostats but should not originate from operations where animals have been vaccinated against coccidia in the previous two flocks. Laboratory strains are not acceptable. Molecular characterisation of the strains should be provided. For inocula used in the AST, the *Eimeria* field strains should ideally undergo one, but in any case, not more than three passage(s) provided virulence is retained.

Based on the current prevalence, mixed inocula should be selected from the following *Eimeria* species:

- chickens – *E. brunetti*, *E. acervulina*, *E. maxima*, *E. mitis*, *E. tenella*, *E. necatrix* and *E. praecox*;
- turkeys – *E. meleagrimitis*, *E. meleagridis*, *E. gallopavonis and E. adenoeides*;
- rabbits – *E. magna*, *E. media*, *E. perforans*, *E. flavescens*, *E. intestinalis*, *E. stiedae*, *E. vejdovskyi*, *E. exiqua*, *E. coecicola*, *E. piriformis* and *E. irresidua*.

For minor poultry species, the most typical *Eimeria* species encountered should be selected.

Virulence titration studies should be performed with each inoculum used in the different studies. The study should include animals in an uninfected untreated control group and multiple groups inoculated with increasing numbers of oocysts. The study follows the principle described for screening tests, considering the age of animals at inoculation and the experimental period and using a small number of animals per group. Virulence is assumed when weight gain is depressed in the experimental period by 25% in chickens and 15% in turkeys and/or intestinal lesion score increased by a minimum of two units on a five-point scale[13] in chickens or a comparable increase in faecal score for turkeys. In addition, mortality/morbidity should be reported.

[13]Five-point scale described in Johnson J. and Reid W.M. (1970): Anticoccidial drugs: Lesion scoring techniques in battery and floor-pen experiments with chickens. Experimental Parasitology 28 (1): 30–36.

For rabbits, no numerical limits can be given for establishing sufficient virulence of an inoculum. However, the same criteria described above can be applied, and results show significant differences used to express a pathogenic dose.

The protocol used in virulence titration studies and the full study report should be submitted.

4 | NUMBER OF IN VIVO EFFICACY STUDIES REQUIRED

The number of independent in vivo efficacy studies required depends on the number of target species/categories for which an application is made.

4.1 | Single animal category

If the application covers only one animal category, the studies required in Section 3 should be performed in that animal category.

4.2 | Multiple categories of the same species of food-producing animals

In principle, conclusions from studies in fattening animals are extended to include animals of the same species that are reared for reproduction, e.g. from chickens for fattening to chickens reared for laying/breeding, from turkeys for fattening to turkeys reared for breeding.

Conclusions from studies in weaned piglets are taken to include suckling piglets for the period in which solid feed is given.

The conclusions on efficacy cannot generally be extended to other categories of the same species at different production stages (e.g. from chickens for fattening to laying hens) unless the claimed effect can be reasonably presumed to be the same between categories. In the latter case, when efficacy has been demonstrated in one category, one additional study in the other category should be provided to establish the minimum efficacious level.

4.3 | Multiple species of food-producing animals

When the application covers several target species, it is recognised that it may be unrealistic to expect studies in all potential target species for which the application is addressed. Therefore, inter-species extrapolation of data can be applied.

In principle, data can be extrapolated between physiologically similar species (Table 4). The degree to which species are physiologically related is judged predominantly in terms of gastrointestinal function. Similarities in metabolism are also considered. However, the interspecies extrapolation can only be applied in case the animals are kept for the same purpose (i.e. meat production or reproduction – including milk or egg production), the mode of action can reasonably be presumed to be the same between species, and the same effects are claimed.

TABLE 4 Extrapolation of efficacy data from certain species to other physiologically related species.

From	To physiologically related species
Chickens for fattening	Other poultry for fattening or reared for reproduction (e.g. turkeys, ducks, geese, pheasants, quail, guinea fowl)
Laying hens	Other birds kept for egg production or breeding[a] (e.g. turkeys, ducks, geese, pheasants, quail, guinea fowl)
Piglets[b] or pigs for fattening	Other porcine species for fattening and reared for reproduction
Sows	Other reproductive porcine species
Calves or cattle for fattening	Other bovines, ovines, caprines, cervids and camelids at the corresponding developmental stage
Dairy cows	Other dairy bovines, ovines, caprines, cervids and camelids
Horses	Other equines
Rabbits	Other leporids

[a]Limited to the effects demonstrated in the laying hens.

[b]Piglets: either weaned piglets or suckling and weaned piglets.

The minimum efficacious level in the physiologically related species would be the same as established in the species/category from which data is extrapolated.

When the application covers multiple species/categories of food-producing animals and the intended effect claimed is the same, the minimum number of independent studies showing the intended effect is shown in Table 5.

TABLE 5 Minimum number of independent studies and target species required for the assessment of efficacy in applications covering multiple species/categories of food-producing animals.

Application for	Number of studies required and species
All poultry for fattening and reared for reproduction	3 in chickens for fattening **or** 2 in chickens for fattening + 1 in turkeys for fattening
All poultry	2 in chickens for fattening + 2 in laying hens
All porcine species for fattening and reared for reproduction	3 covering both weaned piglets and pigs for fattening[a]
All porcine species	1 in weaned piglets + 1 in pigs for fattening + 2 in sows
All bovines, ovines, caprines, cervids and camelids for fattening and reared for reproduction	3 covering both calves and cattle for fattening[a] **or** 1 in calves + 1 in cattle for fattening + 1 in lambs or kids
All dairy bovines, ovines, caprines, cervids and camelids	3 in dairy cows **or** 2 in dairy cows + 1 in dairy sheep or dairy goat
All bovines, ovines, caprines, cervids and camelids	1 in calves + 1 in cattle for fattening + 2 in dairy cows
All rabbits	3 covering both growing and reproductive animals[a]
All insects	2 in honeybees + 2 in other insect species (one in each)
All insects except honeybees	3 in different species (one in each)
All terrestrial species	3 covering both chickens for fattening and laying hens + 3 covering both weaned piglets or pigs for fattening and sows + 3 covering both calves or cattle for fattening and dairy cows (if insects are included) + 3 covering both honeybees and other insect species
All fin fish	2 in salmonids (salmon or trout) + 2 in other fin fish species (one in each)
All crustaceans	3 in shrimp or other crustaceans
All aquatic species	1 in salmonids + 2 in other fin fish species (one in each) + 1 in crustacean or mollusc

[a]At least one study in each category should be performed.

For applications covering all food-producing animal species, efficacy should be demonstrated in species with different digestive systems. Therefore, studies should be provided to support efficacy for all terrestrial (including insects, if relevant) and aquatic species, according to Table 5.

For certain types of additives, the requirements for efficacy studies above may be modified:

- For substances for reduction of the contamination of feed by mycotoxins, radionuclide binders and nutritional additives, the number of studies and the target species are given in Section 3.
- For coccidiostats and histomonostats, specific studies are required for chickens and turkeys. For those intended to be used in minor species, if efficacy has been demonstrated in a major species, then one additional study (preferably a floor pen study) incorporating the most typical *Eimeria* species encountered should be provided for each additional species to a maximum of three.
- For enzymes (as zootechnical additives), there is the possibility of replacing the requirement of three in vivo studies in one category of the same species with a combination of in vitro studies (following validated systems for the demonstration of the intended effect in the animal) in a relevant range of compound feeds plus one in vivo study in each of the target species scope of the application. In those cases, the mode of action can be generally considered similar in all categories of the same species. Therefore, when the additive is intended to be used in all food-producing species,

efficacy should be demonstrated in vivo in four major species (at least one study in each) representing different digestive systems (i.e. chickens, pigs, cattle and salmonids).

4.4 | Pets and other non-food-producing animals

The requirements for the different categories/functional groups of additives apply.

- For additives for which efficacy has been demonstrated in a food-producing animal species and the intended effect is the same, one in vivo study is required for each target pet/non-food-producing species with a maximum of three species in total. For additives intended to be used in ornamental birds, no further studies are required if efficacy has already been demonstrated in chickens for fattening and/or laying hens. For additives intended to be used in ornamental fish, no further studies are required if efficacy has already been demonstrated in salmonids or all fin fish.
- Where the intended effect in the pet/non-food-producing species is different from that described for the food-producing animal species or when efficacy has not been demonstrated in food-producing animal species:

  - When the application is only for one species, three in vivo studies in that species are required.
  - When the application covers two different species (e.g. for cats and dogs), four studies covering both species are required.
  - When the application covers "all pets/non-food-producing animals", four studies covering cats and dogs, plus one additional study in another animal species, are required.

5 | IN VIVO EFFICACY STUDIES

In vivo animal studies are foreseen for all additives exerting the intended effect in the target species. Generally, zootechnical parameters (e.g. growth, milk yield, laying performance, carcass composition, reproduction performance) can only be reliably measured in long-term efficacy studies. In contrast, short-term studies may better demonstrate effects on other parameters (e.g. absorption, digestibility, excretion, retention). The choice of short- or long-term studies or a combination of both will depend on the effect and/or mode of action of the additive.

Such experiments should use numbers and species/categories of animals appropriate to the conditions of use proposed. Studies should be designed to demonstrate the efficacy of the lowest use level of the additive by targeting sensitive and specific parameters, usually in comparison to an appropriate control group. No single design is recommended; flexibility is provided to allow for scientific discretion in the design and conduct of the studies.

As stated in Regulation (EC) No 429/2008, studies must permit the evaluation of the efficacy of the additive according to common farming practices in the EU. The FEEDAP Panel acknowledges that multiple factors, such as location, diet, time, animal breeds, may influence the outcome of the studies beyond the supplementation with the additive. Therefore, when multiple in vivo studies are required to demonstrate the efficacy of the additive, the applicant should ensure that the design and conduct of the different studies take into account these and other relevant factors to reflect the variability in the response.[14]

The experimental design must be justified according to the additive function, use, animal species and category. The trials should be conducted so that the animal health and husbandry conditions do not adversely affect the interpretation of the results. The positive and negative effects should be described for each experiment. Trials should follow the criteria established by recognised externally audited quality assurance schemes (e.g. good laboratory practice, good clinical practice). Evidence should be provided that the work was done by qualified personnel using appropriate facilities and equipment, with a named study director responsible for the research. Studies conducted outside the EU must follow the same quality standards.

Studies involving animals should respect the rules on animal welfare laid down by the EU legislation, particularly those listed in Directive 63/2010/EU. For that purpose, the approval by a competent authority or independent ethical committee, clearly declaring compliance with the animal welfare requirements, should be documented (also for studies conducted outside the EU). It is noted that, according to Directive 63/2010/EC, when the aim of the project requires that the animals are kept under conditions similar to those commonly observed in commercial farms, the husbandry conditions should meet the requirements of Directive 98/58/EC[15] as well as, where relevant, the species-specific legislation.[16]

[14]For example, studies performed in the same location/facilities, during simultaneous (or overlapping) time periods, and using similar diets may be considered as replicates of the same study. In those cases, data will be requested to be pooled and results analysed as a single study.

[15]Council Directive 98/58/EC of 20 July 1998 concerning the protection of animals kept for farming purposes.

[16]Council Directive 1999/74/EC of 19 July 1999 laying down minimum standards for the protection of laying hens; Council Directive 2007/43/EC of 28 June 2007 laying down minimum rules for the protection of chickens kept for meat production; Council Directive 2008/119/EC of 18 December 2008 laying down minimum standards for the protection of calves; Council Directive 2008/120/EC of 18 December 2008 laying down minimum standards for the protection of pigs.

5.1 | General requirements for the in vivo studies

5.1.1 | Test item

Efficacy studies should be based on the additive(s) for which the application is made or, if not, should still allow conclusions on the additive under application to be drawn. A certificate of analysis of the test item used in the study should be provided.

5.1.2 | Route of delivery

The use of the additive in efficacy studies should respect the proposed conditions of use (e.g. use level, route of administration, number of administrations, duration of administration). For additives intended to be administered in a specific form (e.g. bolus), the studies should allow conclusions to be drawn on the particular mode of delivery.

The oral administration routes are, in principle, considered bioequivalent for additives intended for use in feed and water. Therefore, studies can be conducted in either feed, water or a mixture of both, provided that the exposure of the animal is the same. Otherwise, studies for each route would be required.

If a minimum efficacious level has been established in feed, the corresponding concentration of the additive in water can be derived from feed intake. The same principle would apply when the efficacious level has been established in water. For poultry, pigs and rabbits, the water intake would be 2–3 times the dry matter feed intake. In ruminants and horses, concentrations of an additive cannot be consistently extrapolated from feed to water using a fixed ratio of feed-to-water intake. However, these concentrations can be converted to daily amounts and equally administered via feed or water. Consequently, the conversion of feed concentration to water concentration should be done based on the daily ration and the daily water intake.

The concentration of the active substance(s)/agent(s) in the feed/water should be confirmed by analysis. Evidence of adequate sampling should be provided.

5.1.3 | Experimental groups

The design of an efficacy study includes a minimum of two groups:

– a control group

The feed and water of the control group should normally not contain the additive tested. Where studies are required to demonstrate that the additive contributes to the animal's nutritional requirements, the feed of the control group should contain the nutrient at concentrations marginally below the animals' requirements so that a physiological effect of the supplementation with the additive can be expected. Severely deficient diets compromising animal health status/welfare should be avoided.

– a use-level group

The feed/water of the use level group should generally be supplemented with the additive at the lowest proposed use level. For some additives (e.g. nutritional, some colouring agents), the appropriate level of incorporation may be defined by the diet to which it is applied.

Additional groups with the additive supplemented at different levels or a positive control may be included, as appropriate.

5.1.4 | Animals

Animals used should be healthy and preferably from a homogeneous group. For the purpose of this Guidance, health is considered the absence of disease, which allows normal functioning and behaviour of the animal. In production animals this includes the state allowing optimal productivity, in line with current European commercial production standards. Housing and husbandry conditions should be adequate for the study design and conform to European animal welfare regulations. The health and welfare status of the animals should be monitored by a veterinarian at the beginning and throughout the whole duration of the experiment.

Routine vaccinations across all groups are acceptable, but preventive treatments with antibiotics/antimicrobials before the start of the trial should be avoided.

Any treatments with veterinary medicinal products during the study should not interact with the proposed mode of action of the additive. The acceptability of trials in which animals are treated with veterinary medicinal products will depend on various factors, including the number of animals treated, duration of the treatment, distribution between experimental groups and severity of the disease. The acceptability of these studies will be assessed on a case-by-case basis. Studies with an abnormally high mortality rate (i.e. above current European commercial production standards) will not be accepted.

The recommended age/weight for the different species/categories at the start of the study is detailed in Section 5.2.2.1.

5.1.5 | General endpoints

For all in vivo studies, the following parameters should be reported: clinical observations (including general health status), morbidity/mortality, feed intake, water intake for those additives administered via water, initial and final body weight, and milk/egg/honey production (as appropriate).

Specific endpoints will depend on the nature of the additive and its intended effects. More information can be found in Sections 3 and 5.2.2.2 below.

5.1.6 | Statistical considerations

5.1.6.1 | *Design of the experiment*

The experimental unit is the smallest entity to which a given treatment is applied. If animals are housed in groups (e.g. pen, cage) and all the animals in the pen share the same feed source (and feed intake is not measured individually), then the experimental unit for all parameters is the pen, not the individual animal. The experimental unit would be the individual animal when individual feed intake is registered. In the latter case, if the animals are not all housed in the same pen, the model should reflect both the variability among pens and the individual animals (experimental units) within a pen.

Experimental units allocated to the various experimental groups should not differ in a systematic way. Therefore, a recognised randomisation method should be used to allocate treatments to the experimental unit (e.g. pen, animal). The setting conditions (e.g. temperature, light exposure) should be the same for the various groups, including housing, husbandry and diet/water administration. A randomised block design should preferably be used to control experimental settings such as location within facilities. The same design is also recommended in the case of large experiments to ensure concurrency in measurements/determination of endpoints across treatments. Other designs might also be appropriate, in which case the applicant should justify the rationale for the design chosen.

In case of a significant variability across animals of factors which could influence the outcome of the study, animals should be stratified before being randomly allocated to pens/cages/treatments. These factors may include initial body weight, sex, age, stage of lactation, milk yield, parity and egg production. Overparameterised models, including redundant factors, should be avoided.

A proper method for randomisation should be used to allow allocation concealment (no a priori knowledge of group assignment). In practice, the randomisation process must ensure that the investigator cannot influence the allocation of units to the various groups. It is recommended to implement blinding of the caregivers and investigators, for instance, using a proper codification of the treatment to be administered. Blinding is mandatory in cases where the endpoints are gained by expert judgement (e.g. colour by colour fan, lesion score, pathology, faecal score).

5.1.6.2 | *Sample size*

Statistical considerations should be used to determine the sample size used to evaluate the intended effect(s). The optimal sample size calculation is essential to ensure that the experiment can effectively address the proposed hypothesis while minimising the use of animals in line with the 'reduction' principle of the 3R approach.

The null and alternative hypotheses should be set in light of the problem formulation. Difference testing should be used to confirm statistical superiority or inferiority (i.e. alternative hypothesis stating a difference exists), and tests for non-inferiority should be done for experiments aiming at demonstrating non-inferiority between treated groups and a positive control (e.g. bioequivalence study). Additional considerations need to include:

(i) the magnitude of the effect that the study is designed to detect at the substance's lowest recommended level;
(ii) the expected variability of the effect;
(iii) the expected direction of the effect;
(iv) an adequate statistical power;
(v) the confidence level.

When a difference from control (effect) in only one direction (either positive or negative effect) would lead to a conclusion of efficacy (e.g. increase in body weight), a one-sided test can be used. A two-sided test is recommended in all other cases. The applicant should justify the selection of the endpoints, the expected variability and the relevant magnitude of the effect chosen to determine the sample size.

As a guide, a power greater than or equal to 80% (75% for ruminants, minor species, pets and non-food-producing animals) should be ensured. Generally, when testing differences, a confidence level of 90% is adopted for ruminants, minor species, pets, and non-food-producing animals and 95% for all other animal species and categories.

5.1.6.3 | *Statistical analysis*

The statistical analysis should be performed at the level of the experimental unit using models that allow for the comparison of treated and control groups while controlling for factors that could influence the outcome of the experiment whenever

possible. The class of generalised linear mixed models (McCullagh & Nelder, 1989), known as GLMM, offers a suite of methods flexible enough to fit most experimental settings. Typically, this model includes the treatment and other stratification variables (e.g. age) as fixed factors and blocking factors, if any, as random (e.g. animal/pen location). The response variable is the endpoint under investigation. Under certain conditions, a log or other transformations can be needed to linearise the relationship with the explanatory factors. Data transformations can also be used to align data more closely with the normal distribution, particularly in the case of skewed distributions. Different statistical tests and distributional assumptions could be required depending on the type of response variable (i.e. continuous, quantal, dichotomic). The applicant is requested to assess which is more appropriate and provide the rationale for the choice. An indicator of the quality of fit should always be provided.

The analysis of variance is one of the models included in the GLMM class. When using this method, and different levels for the treatment factor are tested, the analysis of variance should include an orthogonal contrast comparing the control group with the use level. This analysis can be complemented with a post hoc test (e.g. Scheffé, Duncan, Tukey, Bonferroni) assessing the multiple comparisons among factor means and/or with a dose–response analysis.

It is strongly encouraged that enough experimental units/observations be used to apply parametric tests to achieve greater statistical power. However, non-parametric tests can be a valid alternative when in doubt (e.g. the median is assessed instead of the mean, sample size is small, data are potentially non-normally distributed, data to be analysed are ordinal, ranked, skewed or with outliers). When different substances are assessed concurrently using the same control, the statistical evaluation should consider only the control and the groups treated with the additive under assessment.

Pooling data from different studies of comparable design may substitute for a single efficacy study, provided that the interaction treatment x study is not significant (the significance level considered will be that referred to in Section 5.1.6.2).

5.2 | Typology of in vivo studies

5.2.1 | Short-term efficacy studies

Short-term studies are defined as those shorter than the minimum duration given in Section 5.2.2.1. They find particular application in measuring bioavailability/bioequivalence of an additive, intestinal absorption and/or excretion of nutrients or other substances, or for the assessment of feed palatability and colouring potency in food of animal origin. Other short-term efficacy studies with animals may be proposed.

5.2.1.1 | *Bioavailability/bioequivalence studies*

Bioavailability is defined as the absorption/transport of the active substance(s)/metabolite(s) to the target cells/tissue(s) where it exerts a typical function/effect. Depending on the nature of the additive, bioavailability will be evaluated by the corresponding specific endpoints (observable or measurable biological, chemical or functional events).

Bioequivalence assesses the expected in vivo biological equivalence of two additives. If two products are assumed to be bioequivalent, they would be expected to be the same for all relevant effects (needs statistical confirmation by a non-inferiority test). Such studies may be used to demonstrate the level at which a novel form or source of a nutritional additive or an additive which colours food of animal origin can substitute for an equivalent additive already authorised or established, which should provide evidence for affecting the selected endpoint.

5.2.1.2 | *Digestion/balance studies*

The quantitative outcome of a digestion study is the digestibility (e.g. apparent or true, faecal or ileal) of a nutrient as influenced by the additive. Balance studies are preferred because they deliver additional information on quantitative excretion and retention of a nutrient/energy. Digestibility studies in which animals are cannulated are discouraged.

Digestibility/balance studies should be performed considering an adequate period of adaptation to the diet (and experimental conditions). The minimum duration of this pre-period should be at least 7 days for all species, with the exemption of ruminants, for which it should be at least 14 days. The collection period should be at least 5 days for all species.

In laying hens, dairy cows and sows, balance/digestibility studies should include measurements of the product output (e.g. eggs, milk, litter). For applications in sows at any reproductive stage, digestibility studies should be performed in both gestating and lactating sows.

Digestibility studies in aquatic species are discouraged; retention studies analysing body composition should be made instead.

5.2.1.3 | *Palatability studies*

Palatability studies should provide the animal with a free choice of feed (simultaneous access to control and test feed). The experimental design should exclude the possibility that the results are influenced by the position in which the individual feed types are offered. The minimum duration of studies of this type is two periods of 5 days each, with an intermediate period in which only the control feed should be provided.

The two diets should be essentially equal in composition, with the only difference being the presence of the additive in the test diet at the proposed inclusion rate (analytically confirmed).

Feed intake should be recorded at least once daily and reported accordingly.

A similar design should be applied for additives intended to be used in water. In that case, feed and water intake should be monitored.

5.2.2 | Long-term efficacy studies

5.2.2.1 | *Duration of the long-term efficacy studies*

Generally, the duration of efficacy trials should correspond to the application period claimed. The necessary minimum duration of efficacy trials depends on the animal species/category and is reported in Table 6.

TABLE 6 Minimum duration of long-term efficacy studies.

Category	Start of the study	Minimum duration
Chickens for fattening	1 day of age	35 days
Laying hens	< 30 weeks of age and ≥ 90% laying rate	84 days
Turkeys for fattening	1 day of age	84 days
Piglets (weaned)	≤ 7 days after weaning	42 days 35 days if the growth rate is ≥ 0.5 kg/day
Pigs for fattening	≤ 35 kg	Until slaughter, but not less than 70 days
Sows	For effects on reproduction: from insemination/mating For effects on lactation or on piglets: not later than from parturition	For effects on reproduction: two full reproduction cycles For effects on lactation or on piglets: until the end of the weaning period (but not less than 28 days)
Calves	< 6 weeks of age	56 days
Cattle for fattening	Full development of rumination and ≤ 12 months of age	84 days
Dairy cows	< 16 weeks after parturition and Milk yield ≥ 30 kg/day	84 days
Lambs/kids	< 8 weeks of age	56 days
Dairy ewes/goats	< 8 weeks after parturition	84 days
Rabbits (growing)	< 6 weeks of age	42 days
Breeding does	For effects on reproduction: from insemination/mating For effects on kits: no later than from parturition	For effects on reproduction: Two cycles For effects on kits: until the end of the weaning period
Salmon and trout	Trout ≥ 10 g Salmon ≥ 50 g	84 days
Honeybees		28 days
Other insects		Whole production cycle
Cats, dogs and other non-food-producing animals		28 days

For minor species not included in the table above, the duration of the long-term studies should correspond to that of the physiologically related major species listed in Table 4. The minimum duration for all other species/categories should be 42 days for growing animals and 56 days for adult animals.

If an additive is applied for a specific and shorter period than that given in the table above, it should be administered according to the proposed conditions of use. However, the observation period should not be shorter than 28 days and involve the relevant endpoints (e.g. for sows for reproduction, the number of piglets born alive when considering the gestation period, or the number and weight of weaned piglets when considering the lactation period).

5.2.2.2 | *Endpoints*

The endpoints to be measured depend on the effects expected from the additive (see Section 3). A non-exhaustive list of endpoints for some common effects is given below.

Performance parameters and related parameters

For all studies, feed intake, initial and final body weight, body weight gain, feed-to-gain ratio and water intake for those additives administered via water should be provided. Clinical observations should also be monitored, including general health status, morbidity and mortality.

Additional parameters for:

- laying hens: laying rate, egg weight, feed-to-egg mass ratio, egg mass/hen per day.
- breeding hens: laying rate, fertility, hatchability and chick viability.
- dairy animals: milk production (including fat/energy corrected milk), feed efficiency, milk composition (total solids, protein, fat, lactose and urea), protein, fat and lactose yield and somatic cell counts.
- sows/does: number of piglets born, piglets born alive, litter weight at birth and at weaning, number of piglets weaned, weaning to oestrus interval.
- fish/crustaceans: specific growth rate.

Product quality/composition

When measuring changes in the product quality or composition as an intended effect of the additive, the following endpoints can be considered appropriate.

- Composition: e.g. nutrient content
- Physical/technological properties: e.g. water binding capacity, oxidative stability.
- Sensory modification of food products: e.g. colour, taste, smell, texture. The sensory properties of the food products should be measured, preferably by objective methods. However, it is recognised that some parameters can be better assessed using, e.g. a trained panel or other subjective methods.
- Hygiene quality of food products: e.g., numbers of spoilage organisms, potential human or animal enteropathogens. Studies should clearly identify the target microorganisms. These should be enumerated, and their prevalence in faeces/intestinal contents or the carcass should be established. Ideally, the pathogens should be measured in the food products.

Other endpoints may be proposed and justified.

Environmental effects

Direct effects on the environment may include, for example, reduction of methane, ammonia, carbon dioxide emissions and reduction of odour or odorous compounds.

Indirect effects on the environment may result from increased nutrient utilisation and reduced excretion of, e.g. nitrogen, phosphorus and sulphur, if appropriate dietary adjustments are made.

Faecal consistency

It is recommended to use objective measurements such as dry matter content of faeces. Subjective observations of faecal consistency alone are discouraged. If used, continuous subjective observations following recognised methods should be complemented with periodic objective measurements.

Welfare

Effects can be monitored by endpoints related to the physiological, behavioural and/or immunological status of the animal. The endpoints selected should be appropriately justified.

5.3 | Studies on the quality of products when this is not the effect claimed

Evidence should be provided that the additive does not have a negative effect or another unintended effect on sensory and nutritional (and hygienic and technological if appropriate) characteristics of food deriving from animals fed with the highest proposed level of the additive. Evidence can be based on physiological/metabolic considerations or given by reference to published literature. Otherwise, specific studies should be provided. Appropriate endpoints may be found under Section 5.2.2.2.

The omission of these studies should be adequately justified.

6 | IN VITRO STUDIES

For additives affecting the characteristics of feed, efficacy should be demonstrated using laboratory-based studies. Efficacy studies should be based on the additive(s) for which the application is made. A certificate of analysis of the test item used in the study should be provided. The concentration of the active substance(s) or agent(s) in the feeds/water should be confirmed by analysis. The experimental design and methodology used should be appropriate to the intended effects of the additive. Studies should be designed to demonstrate the efficacy of the minimum use level(s) of the additive by targeting sensitive parameters compared to a control group. The study should be designed to cover a representative range of feeds (including feed materials, complete or complementary feed) to which the additive will be applied, including water, if appropriate.

The experimental design should consider sufficient observations to allow an adequate statistical analysis. The results of each test/subset should be statistically evaluated, and a confidence level of 95% should be adopted. It is strongly encouraged that enough experimental units/observations be used to apply parametric tests to achieve greater statistical power. However, non-parametric tests can be a valid alternative when in doubt and, regardless of the outcome of normality tests, should be used when the number of observations per experimental group is small. For in vitro studies, the number of replicates per experimental group should be at least four, even when non-parametric tests are to be applied. When different substances are assessed concurrently using the same control, the statistical evaluation should consider only the control and the groups treated with the additive under assessment.

All trials should follow the criteria established by recognised externally-audited quality assurance schemes (e.g. good laboratory practice or ISO standards). Evidence should be provided that the work was done by qualified personnel using appropriate facilities and equipment and responsible to a named study director.

7 | REPORTING OF EFFICACY STUDIES

For each efficacy study, a report describing the objectives, materials and methods, results and conclusions should be submitted. The original study protocol should be included; any deviations from the original protocol should be clearly indicated and justified in the final report. The reports should include the raw data in editable and accessible digital format and detailed results, including descriptive statistics, statistical tests and model outcomes. Reports for in vivo studies should start with a trial protocol data sheet (Appendix A), followed by the full study report. International units should be used to express the results. The study pre-notification number should be indicated.

It is recommended that the study report follows the structure detailed below and contains the following information. Applicants are encouraged to follow the recommendations of the EFSA Guidance on statistical reporting (EFSA, 2014).

Title: The title should provide a concise and precise description of the study, including the type of study, the product under assessment and the animal species/category.

Summary: The summary should include the objectives, a description of the design and methods, the principal results and the conclusions of the study.

Objectives: The objectives of the study should be clearly described.

Materials and methods: methods, apparatus and materials used, details of the species, breed or strain of the animals, their number and the conditions under which they were housed and fed. In particular, the following should be recorded and reported:

Ethical statement

1. Certificate of approval of the study protocol by a competent authority or independent animal welfare committee (including number/code of authorisation) clearly declaring compliance with the animal welfare requirements, according to EU legislation.

Animals, housing and husbandry

2. Animals: species (for aquatic species intended for human consumption: identification should be made by their colloquial name followed in parenthesis by the Latin binomial), breed, age (and size/length for aquatic species), initial body weight, sex, identification procedure, physiological stage and general health certified by a veterinarian.
3. Husbandry conditions: method of feeding (ad libitum/restricted) and rearing conditions (pen/tank size, stocking density, temperature, bedding/floor characteristics, light cycle and intensity, environmental enrichment); for aquatic species water quality including water flow rate, water temperature and salinity, where relevant.
4. Diets: description of manufacture and quantitative composition of the diet(s) in terms of ingredients used (including premixes), relevant nutrients (calculated and analysed values) and energy (digestible, metabolisable or net). The certificates of analysis of the proximate composition of the diets should be provided. In addition, for studies with enzymes, the diets should be analysed for the enzyme-specific substrate (e.g. non-starch polysaccharides, phytate-P).

Study design

5. Study location, dates and responsible individuals.
6. Study duration.
7. The study design type (e.g. factorial, stratified, cross-over).
8. Experimental groups: number of treatment and control groups, number of replicates (experimental unit) per group and number of animals per replicate.
9. The experimental unit (e.g. individual animal, pen) should be indicated.
10. The basis for the different measurements (e.g. individual animal, pen) should be indicated for each parameter measured.
11. Rationale for selecting the number of animals/replicates used (sample size calculation). Power analysis should be provided.
12. Steps taken to minimise bias, including randomisation and blinding (see Section 5.1.1 of the EFSA Guidance on statistical reporting (EFSA, 2014)).
13. Test item: intended concentration of the active substance(s) or agent(s) in the feeds. The method of analysis used should be reported.

Experimental procedures

14. The procedures for the different experimental groups should be detailed, including the frequency and methodology of measuring the parameters/endpoints recorded.
15. The health of the animals should be monitored, and the morbidity and mortality (including culling) recorded.
16. The methodology to correct the feed-to-gain ratio for mortality (including culling) should be reported.

Statistical methods

17. The result of the power analysis should be reported.
18. The methods to perform statistical analysis should be stated, including those used to identify outliers and handle missing data. A justification should be given if any relevant data points are excluded from the model (e.g. outliers).
19. Describe any methods used to assess whether the data meet the assumptions of the statistical approach.

Results: Results should be presented for all endpoints considered in the study. Tables should be used to summarise the results from treatments. For all endpoints measured on individual animals in a pen, a summary parameter of the endpoint in the experimental unit should be used (e.g. mean for continuous measures such as body weight, median and counts for quantal measurements such as severity of an outcome or mortality). Summary parameters should always be adjusted for losses (mortality/culling). The distribution of losses within the treatment groups should be assessed to avoid the risk of introducing a bias.

20. Health status of the animals, morbidity and mortality, including culling. The timing and prevalence of any unexpected/undesirable incident/effect in individuals or groups. Therapeutic/preventive treatments, if any, must be recorded. The likely cause of death and/or reason for culling should be established by a veterinarian and reported (including the necropsy report, where relevant).
21. The report should include data from all animals or experimental units involved in the trials. Cases that cannot be assessed due to a lack or loss of data should be reported, and their distribution within the groups of animals should be indicated.
22. The concentration of the active substance(s) or agent(s) in the feeds to which the additive is added should be analysed and reported. When multiple test feeds are produced (e.g. due to multistage feeding), every batch of feed needs to be analysed. A certificate of analysis of the test item used in the study should be provided.
23. Report the results for each endpoint measured/analysis carried out, with a measure of precision (e.g. standard error or confidence interval).
24. The report should include descriptive statistics plus the detailed outcome of any statistical analysis performed for all measured endpoints at each time point and the overall duration of the experiment (if relevant).
25. The measurement units should be specified for any result reported.

Discussion

26. The interpretation of the results, considering the study objectives and hypotheses and other relevant studies in the literature, if relevant.
27. Comments on the study limitations, including any potential sources of bias, any limitations of the animal model and the imprecision associated with the results, if relevant.

Conclusions

28. The conclusions from the study should be drawn considering the objectives, the hypothesis and the outcome of the study.

Raw data, certificates of analysis

29. The raw data should be provided in the form of an electronic database, in an editable and accessible format (e.g. Excel), and accompanied by a data dictionary containing the description of the variables and the metadata needed to analyse them properly.
30. All codes, logs and complete outputs for the final statistical analysis (i.e. the results and analysis reported) should be provided in an electronic and readable format.
31. The report should include the certificates for the different analyses performed and the reports of the veterinary observations (including gross pathology and histopathology, haematology and clinical chemistry, when appropriate).

Reports of in vitro studies should respect the principles described above, as appropriate.

8 | LITERATURE STUDIES

Reference can be made to published studies to support the efficacy of the additive (including the demonstration of compatibility with other additives or components of the diet). The additive (active substance(s)/agent(s)) in literature studies should be identical to that under application or, if not, should still allow conclusions on the additive under application to be drawn. The concentration of the additive (active substance(s)/agent(s)) in the feed should reflect the conditions of use specified in the application. The target species covered in the literature studies should be relevant to the application. The application level, replicates, duration and measured endpoints should align with the requirements listed in this Guidance and allow a conclusion on the efficacy of the additive

The list of relevant references should be compiled in a reference management software and provided in .RIS format. Copies of the relevant papers should be provided. The applicant must ensure that the terms and conditions asserted by any copyright holder of publications or information submitted to EFSA are fully satisfied. The applicant should consult with copyright licensing authorities (i.e. at national level) for Guidance on purchasing copyright licences to reproduce any publications provided to EFSA. The applicant remains solely responsible and liable for obtaining all necessary authorisations and rights to use, reproduce and share the publications provided to EFSA.

ABBREVIATIONS

AST	anticoccidial sensitivity test
CFU	colony forming unit
DM	dry matter
DON	deoxynivalenol
EURL	European Union Reference Laboratory
FEEDAP	EFSA Scientific Panel on Additives and Products or Substances used in Animal Feed
GLMM	general linearised mixed model
ISO	International Organization for Standardization
IT	infected treated
IUC	infected untreated control
UT	uninfected treated
UUC	uninfected untreated control

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CONFLICT OF INTEREST

If you wish to access the declaration of interests of any expert contributing to an EFSA scientific assessment, please contact interestmanagement@efsa.europa.eu.

REQUESTOR

European Commission

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APPENDIX A

Trial Protocol datasheet

Identification of the additive:	Batch number:
Trial ID:	Location:
Start date and exact duration of the study:	**Pre-notification number:**
Number of treatment groups (+ control(s)):	Replicates per group:
Total number of animals:	Animals per replicate:
Concentration(s) of the additive/active substance(s)/agent(s) (mg or Units of activity or CFU/kg complete feed or L water)	
Intended:	Analysed:
▪	
Substances used for comparative purposes:	
Intended concentration:	Analysed:
Animal species/category:	Latin binomial (for fish):
Breed:	Identification procedure:
Sex: Age at start:	Body weight at start:
Physiological stage:	General health:
Fork length at start (for fish):	
Lighting conditions:	Pen/cage/tank dimensions :
Water quality, including temperature, salinity, O_2 and CO_2 (for fish):	Environmental enrichment :
Diets (type(s)):	
Presentation of the diet: Mash ☐ Pellet ☐ Extruded ☐ Other	
Composition (main feed materials):	
Nutrient content (relevant nutrients and energy content)	
Intended values:	
Analysed values:	
Date and nature of the examinations performed:	
Method(s) of statistical evaluation used:	
Therapeutic/preventive treatments (reason, timing, kind, duration):	
Timing and prevalence of any undesirable consequences of treatment:	
Date	Signature Study Director

▪ In case the concentration of the additive in complete feed/water may reflect insufficient accuracy, the dose of the additive can be given per animal/day or mg/kg body weight or as a concentration in the complementary feed.